Dentin Matrix Protein 1 Enhances Invasion Potential of Colon Cancer Cells by Bridging Matrix Metalloproteinase-9 to Integrins and CD44

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Abstract

The up-regulation of various matrix metalloproteinases (MMP), certain cell receptors such as integrins and CD44, and the SIBLING family of integrin-binding glycophosphoproteins have been reported separately and in various combinations for many types of tumors. The mechanisms by which these different proteins may be interacting and enhancing the ability of a cancer cell to survive and metastasize have become an interesting issue in cancer biology. Dentin matrix protein 1 (DMP1) has been known for a number of years to bind to CD44 and ArgGlyAsp sequence–dependent integrins. This SIBLING was recently shown to be able to specifically bind and activate proMMP-9 and to make MMP-9 much less sensitive to inhibition by tissue inhibitors of metalloproteinases and synthetic inhibitors. In this study, we used a modified Boyden chamber assay to show that DMP1 enhanced the invasiveness of the MMP-9 expressing colon cancer cell line, SW480, through Matrigel in a dose-dependant manner. DMP1 (100 nmol/L) increased invasion 4-fold over controls (86.1 ± 13.9 versus 22.3 ± 9.8, $P < 0.001$). The enhanced invasive potential required the presence of MMP-9 and at least one of the cell surface receptors, CD44, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ integrin. The bridging of MMP-9 to the cell surface receptors was shown by both pull-down and fluorescence activated cell sorting experiments. Because all of these proteins were also shown by immunohistochemistry to be expressed in serial sections of a colon adenocarcinoma, we have hypothesized that the MMP-9/DMP1/cell surface complexes observed to enhance cell invasion *in vitro* may be aiding metastatic events *in vivo*. (Cancer Res 2005; 65(24): 11545-52)

Introduction

Dentin matrix protein 1 (DMP1) is an acidic phosphoglycoprotein and member of the integrin-binding SIBLING protein family (1). All of the SIBLINGs have been shown over the years to be up-regulated in many different primary tumors but curiously are often not expressed in high-passage cell lines derived from these tumors. DMP1, historically thought to be expressed in only bones and teeth, has recently been shown to be made in some normal ductal epithelial tissues as well (2, 3). Although the precise functions of the protein have not been explicated, it has been associated with the regulation of postnatal chondrogenesis and subsequent osteogenesis and matrix mineralization (2, 4–6). DMP1 has been shown

to bind both $\alpha_v\beta_3$ integrin and CD44 cell surface molecules (7). Several studies have shown significantly elevated expression of DMP1 in a number of cancerous tissues, including lung (8), breast, uterus, thyroid, and colon (9). DMP1 has been shown to bind with nanomolar affinity and to activate latent matrix metalloproteinase-9 (proMMP-9) apparently without removal of its propeptide. It can bind active MMP-9 without changing the ability of protease to digest natural substrates and causes the protease to remain active in the presence of normally inhibitory amounts of tissue inhibitors of metalloproteinase 1 (TIMP1) *in vitro* (10). Up-regulation of MMP-9 has been described for many types of cancers, including colon cancer (11), and it has been used in a portal blood assay to determine colorectal metastases in liver (12). Furthermore, the increased expression of MMP-9 in primary colon tumors has been associated with liver metastases (13) and was increased in biopsy samples from patients with colon cancer (14). We have recently shown by array analysis that there is a coordinate increase in MMP-9 and DMP1 expression in lung and kidney cancer (9). The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have also been associated with colon cancer metastasizing to liver in an orthotopic murine model (15). CD44 expression has been linked to transendothelial migration of colon cancer cell lines (16) and to the invasion of colorectal cancer cell lines that also showed increased MMP-9 expression (17). Another member of the SIBLING family, bone sialoprotein (BSP), promotes invasion of several osteotropic cancer cell lines by localizing MMP-2 on the cell surface through $\alpha_v\beta_3$ integrin (18). DMP1 had no effect on the invasion of the recombinant BSP-responsive cancer cell lines, suggesting that MMP-2 was the rate-limiting protease for those particular cell lines in the modified Boyden chamber assay.

MMPs are a class of zinc-dependent endopeptidases that are collectively capable of digesting all extracellular matrix components. These proteases play an important role in remodeling the extracellular matrix and their regulation is implicated in a variety of diseases, including cancer invasion and metastasis (19). The regulation of MMP activity in tumor cell invasion and metastasis involves selective expression as well as a balance of activators, inhibitors, and regulators. The first step in invasion generally requires digestion of basement membrane proteins, including type IV collagen. MMP-2, MMP-3, MMP-7, MMP-9, and MMP-10 are all able to degrade this collagen (20). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have long been known to degrade extracellular matrix macromolecules in basement membranes and other interstitial connective tissues (21). It has been shown that the localization of active MMP-9 on the cell membrane of the invasive cell through association with CD44 can be critical for tumor cell invasion and metastasis (22). Similarly, MMP-2 localization on the cancer cell surfaces through $\alpha_v\beta_3$ integrin has been reported to be important in the initiation of invasion of several cancer cells (18, 23, 24). Therefore, understanding the interaction of MMPs, cell adhesion molecules, and any modifying proteins could offer insight into cancer biology.

We hypothesized that DMP1 may play a role in cancer metastasis by bridging MMP-9 to the cell surface through $\alpha_v\beta_3$ integrin, $\alpha_v\beta_5$ integrin, and/or CD44. To test this hypothesis, both fluorescence-activated cell sorting (FACS) analysis and a previously described modified Boyden chamber invasion assay were used (25) to measure the enhancement of invasion by DMP1 for the colon cancer cell line, SW480, cells known to expresses significant amounts of MMP-9 *in vitro*.

Materials and Methods

Materials. The human colon cancer cell line SW480 (CCL-228) was obtained from the American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was purchased from Equitech-Bio, Inc. (Kerrville, TX). RPMI 1640, L-glutamine, 2-mercaptoethanol, sodium pyruvate, modified Eagle's medium (MEM) nonessential amino acids, HBSS, PBS, Versene (0.53 mmol/L EDTA in PBS), and 10% zymogram gelatin gels were from Invitrogen, Inc. (Carlsbad, CA). Matrigel was from Collaborative Research, Inc. (Bedford, MA). Transwell inserts and companion plates were purchased from BD Biosciences Discovery Labware (Bedford, MA). Calcein acetoxymethyl ester dye and the Alexa Fluor 488 protein-labeling kit were purchased from Molecular Probes, Inc. (Eugene, OR). Mouse anti-human $\alpha_v\beta_3$ integrin monoclonal antibody (mAb) immobilized on immunoaffinity gel matrix (GEM1976), mouse antihuman $\alpha_v\beta_5$ integrin mAb immobilized on immunoaffinity gel matrix (GEM1961), antihuman $\alpha_v\beta_3$ integrin mAb (LM609, MAB1976Z), antihuman $\alpha_v\beta_5$ integrin mAb (P1F6, MAB1961Z), recombinant $\alpha_v\beta_3$ integrin in Triton X-100 (CC1018), and recombinant $\alpha_v\beta_5$ integrin in Triton X-100 (CC1025) were obtained from Chemicon International, Inc. (Temecula, CA). Antihuman CD44 mAb (BU75) was purchased from Ancell Corporation (Bayport, MN). ProMMP-9 was from Oncogene Research Products (Boston, MA). ProbeQuant G-50 micro-columns were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Microcon YM-30 centrifugal filter devices were from Millipore Corporation (Bedford, MA).

Cell culture. The SW480 cells were first grown in RPMI 1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 5 mmol/L 2-mercaptoethanol, 2 mmol/L sodium pyruvate, and 0.1 mmol/L MEM nonessential amino acids in a humidified atmosphere of 5% CO_2/95% air at 37°C. When the cells were ~80% confluent, they were either used in the experiments as described in appropriate sections below or subcultured with the split ratio of 1:10 for up to 20 passages.

SIBLING production and purification. Recombinant DMP1 with good posttranslational modifications (including glycosylation, sulfation and, possibly phosphorylation) was made as described previously (7). Briefly, full-length bovine DMP1 cDNA (26) was subcloned into a high-expression, replication-deficient adenovirus type 5 with transcription under the control of the cytomegalovirus promoter. The DMP1-LysAlaGlu (KAE) construct was made by *in situ* mutagenesis of the ArgGlyAsp (RGD) domain into KAE while in pBluescript. The entire insert was checked for fidelity and then shuttled to the adenovirus plasmid (27). The adenoviruses were selected, purified, and expressed as previously described (28). Recombinant SIBLINGs were generated by infecting mid-passage, subconfluent normal human bone marrow stromal cells (a gift of Dr. Pamela Gehron Robey, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD). Harvested serum-free medium was subjected to anion-exchange chromatography, as described (7, 28), to isolate SIBLINGs. Purity of each SIBLING was >95%, as measured after SDS-PAGE.

Preparation of Matrigel-coated inserts. Matrigel-coated inserts placed in 24-well plates were prepared as described previously (18). Briefly, Matrigel was diluted with ice-cold sterile H_2O and mixed well. The solution was dispensed as 5 μg/50 μL/insert/well. The plates were left in a laminar flow tissue culture hood overnight to dry. The inserts were then rinsed with sterile HBSS to rehydrate films and the plates were immediately used as described below.

Modified Boyden chamber cell invasion assay. Invasiveness of each cancer cell line was measured by using an UV-opaque transwell polycarbonate membrane (diameter of 6.4 mm and pore size of 8 μm) in a modified Boyden chamber cell invasion assay. Transwell inserts precoated with Matrigel as described above were placed in a 24-well plate. Preconfluent cells were removed from culture dishes with 0.53 mmol/L EDTA in PBS, washed twice in HBSS, and resuspended in serum-free RPMI 1640 at a final density of 4×10^5 cells/mL. Quadruplicate cultures of cells were briefly pretreated in a final volume of 250 μL of serum-free medium [containing 0.1% bovine serum albumin (BSA)] with either buffer or SIBLINGs in sterile 1.5 mL microcentrifuge tubes for 10 minutes and then placed in the upper compartment of a Boyden chamber. In some cases, cells were first treated for 20 minutes with blocking antibodies or control IgGs in the tube and then placed in the upper chamber. In the latter cases, buffer or recombinant SIBLING was then added directly to the chamber. To induce migration through the Matrigel layer, the lower chambers were filled with 750 μL of serum-free medium conditioned by mouse NIH 3T3 fibroblastic cells and containing 0.1% BSA. Cells were then incubated in humidified incubator at 37°C for 24 hours. Cells that had not migrated through the barrier were removed from the top compartment and inserts were moved to another 24-well plate in which each well contained 0.5 mL of the fluorescent dye, calcein acetoxymethyl ester, at 4 μg/mL. The plate was incubated at 37°C for 45 minutes to allow the living cells to take up and activate the dye before the fluorescent intensity was read from the bottom of the insert with a fluorescence plate reader (Wallac 1420 VICTOR[2] Multilabel Reader, Perkin-Elmer Life Sciences, Inc., Boston, MA). Fluorescence intensity was proportional to the number of cells having migrated to the bottom of the UV-opaque membrane.

Pull-down experiments. Commercial mouse anti-human $\alpha_v\beta_3$ integrin or $\alpha_v\beta_5$ integrin mAb immobilized on immunoaffinity gel matrix beads were washed thrice in ice-cold Triton buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.2% Triton X-100, 2 mmol/L $MgCl_2$, and 0.1 mmol/L $CaCl_2$] and incubated in 1 mL Triton buffer containing 1% BSA at 4°C for 30 minutes with gentle shaking. After washing thrice with 1 mL of ice-cold Triton buffer, the beads were gently shaken with or without 10 μg of $\alpha_v\beta_3$ integrin or $\alpha_v\beta_5$ integrin complex in 50 μL Triton buffer at 4°C for 10 minutes. The beads were then gently pelleted, the liquid was carefully removed, and the beads were washed in 1 mL Triton buffer. The beads were then resuspended in 1 mL buffer and separated into equal volume aliquots. An aliquot was gently shaken with buffer alone or buffer containing 500 nmol/L DMP1 or 500 nmol/L DMP1-KAE (in a final volume of 50 μL) at 4°C for 10 minutes. The beads were then pelleted, washed in 1 mL of Triton buffer, and incubated in 50 μL of Triton buffer containing 1 μg of proMMP-9 at 4°C for 10 minutes. The beads were pelleted and washed with 1 mL Triton buffer, and the MMP-9 was eluted from the beads with 80 μL of 1× zymogram sample buffer [2.5 mL of 0.5 mol/L Tris-HCl (pH 6.8) + 2 mL glycerol + 4 mL of 10% (w/v) SDS + 0.5 mL of 0.1% bromophenol blue adjusted to 20 mL with distilled water] and resolved by electrophoresis on a 10% (gelatin) zymogram gel.

SDS-PAGE and zymography. Samples in zymogram gel sample buffer were loaded on a 10% (gelatin) zymogram gel, subjected to electrophoresis, and processed as recommended by the manufacturer. Resulting Coomassie-stained gels were visualized by dynamic integrated exposure using an EagleEye II imaging system (Stratagene Corp., La Jolla, CA).

Labeling of purified human matrix metalloproteinase-9 with Alexa Fluor 488 dye. Latent protease (proMMP-9) was fluorescently labeled with the Alexa Fluor 488 protein-labeling kit according to the protocol of the manufacturer but adjusted to the smaller amount of protein being labeled. All steps were done at 4°C. Briefly, shipping buffer from 50 μg of proMMP-9 was changed into the reaction buffer (PBS) on ProbeQuant G-50 microcolumns, and the resulting eluant concentrated to ~50 μL with a prewashed Microcon YM-30 centrifugal filter device. Five micro-liters of 0.1 mol/L sodium bicarbonate were added to raise the pH to 7.5 to 8.5 for efficient labeling. The reactive dye was dissolved in 0.5 mL of PBS containing 0.1 mol/L sodium bicarbonate, 50 μL of Alexa Fluor 488 dye was added to the proMMP-9 solution, and the reaction mixture was stirred for 2 hours. The labeled proMMP-9 protein was then separated from the unreacted dye on a ProbeQuant G-50 microcolumn (in PBS) and stored as aliquots at −80°C until use.

Flow cytometry. Cells were detached from culture dishes with PBS containing 0.53 mmol/L EDTA, washed twice in HBSS, and then incubated at 2×10^6/mL at room temperature for 10 minutes with buffer alone or buffer containing either 500 nmol/L DMP1 or 500 nmol/L DMP1-KAE. For the studies involving the blocking antibodies, cells were incubated with buffer alone or buffer containing $\alpha_v\beta_3$ integrin antibody (LM609, 4 μg/mL), $\alpha_v\beta_5$ integrin antibody (P1F6, 4 μg/mL), CD44 antibody (BU75, 4 μg/mL), or control IgG (4 μg/mL) at room temperature for 10 minutes, and then the mixture was incubated with 500 nmol/L DMP1 or 500 nmol/L DMP1-KAE for another 10 minutes. In the final step for all samples, cells were pelleted, washed once in HBSS, and then incubated at room temperature with Alexa Fluor 488–labeled human proMMP-9 at 5 μg/mL for 10 minutes. The cells were pelleted, washed once, resuspended in HBSS, and analyzed immediately with a FACSCalibur cell sorter equipped with a 488 nm argon laser and Cellquest software (BD PharMingen, Bedford, MA).

Rabbit anti-human matrix metalloproteinase-9 polyclonal antibody (LF-184) production. The carboxy-terminal 23 amino acids of human MMP-9 (RSELNQVDQVGYVTYDILQCPED), a region with relatively low homology to human MMP-2, was synthesized and conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce Biotechnology, Inc., Rockford, IL) through the cysteinyl residue. A New Zealand white rabbit was injected four times with ∼1.0 mg of conjugate under an approved animal protocol at a contract facility (Covance, Inc., Denver, PA). ELISA showed good activity to authentic human MMP-9 but no cross-reactivity with authentic human MMP-2 or MMP-3. Synthetic peptide (1.6 mg) was conjugated to 2 mL column of SulfoLink agarose beads (Pierce Biotechnology) according to the instructions of the manufacturer. Antibodies were bound to the affinity column, washed sequentially with PBS, 0.05% Tween 20 in PBS (PBS-T), PBS, and then eluted in 0.1 mol/L glycine (pH 2.4). The fractions were immediately neutralized, dialyzed against PBS, and stored frozen at −80°C until use.

Immunohistochemistry. Immunohistochemistry was done on human colon adenocarcinoma serial sections cut from blocks obtained from the Cooperative Human Tissue Network, Mid-Atlantic Division (Charlottesville, VA) under Institutional Review Board approval. The Zymed ST5050 automated system (Zymed Lab, Inc., San Francisco, CA) was used with following primary antibodies: mouse anti-human DMP1 mAb (LFMb-31, 1:100; ref. 2), mouse anti-human CD44 (H-CAM, 1:100) mAb (MAB2140, Chemicon International), mouse anti-human α_v integrin (CD51, 1:50) mAb (407286), mouse anti-human β_3 integrin (CD61, 1:50) mAb (407314), mouse anti-human β_5 integrin mAb (407316, 1:50; all from EMD Biosciences, Inc., San Diego, CA), and the affinity purified rabbit anti-human MMP-9 polyclonal antibody (LF-184, 1:200). Briefly, the paraffin block containing a moderately differentiated colon adenocarcinoma was serially sectioned (5 μm thickness) and dried on silane-coated slides (Statlab Medical Products, Inc., Lewisville, TX). The sections were manually deparaffinized in xylene and rehydrated through a graded ethanol series (100%, 95%, and 70%). After the endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 minutes, the sections were washed thrice in PBS for 5 minutes each and covered with PBS-T before loading the slides onto the preprogrammed ST5050 automated immunohistochemistry machine. The ST5050 was programmed to incubate each slide for 1 hour with appropriate primary antibody diluted in 10% normal goat serum in PBS. The slides thereafter went through a 4×1 minute wash cycle with PBS-T before incubation with SuperPicTure Polymer horseradish peroxidase (HRP)–conjugated broad-spectrum secondary antibody (Zymed Lab) for 10 minutes. The slides were processed through another wash cycle and then were developed with AEC Single Solution chromogen (Zymed Lab) for 2 minutes. Sections were lightly counterstained manually with Mayer's hematoxylin for 10 to 20 seconds before applying an overlay of Clearmount glaze (both from Zymed Lab) over the sections. After air drying, slides were coverslipped with Histomount (Zymed Lab). All steps were done at room temperature. Negative controls included the substitution of primary antibody with nonimmune rabbit serum or mouse IgG control (Zymed Lab). Sections were photographed with an AxioCam MR-MRGrab camera imaging system (Carl Zeiss Vision, Munich, Germany), which included an Axioplan2 microscope, an AxioCam MRm camera, and AxioVision 3.1 software.

Statistical analysis. Data are the mean of quadruplicate determinations and 95% confidence interval. Each experiment has been repeated at least twice. In each case, data from a single representative experiment are shown. Multiple comparisons were done with a one-way ANOVA followed by Dunnett's test for treatment versus control comparisons. Pairwise comparisons were done by performing nonparametric Mann-Whitney U test. In each analysis, differences were considered statistically significant for $P < 0.05$. All statistical tests were two sided.

Results

Dentin matrix protein 1 enhances invasiveness of colon cancer cell line, SW480, *in vitro*. Previous work showed that many human cancer cell lines that produced MMP-2 showed enhanced invasion by BSP/MMP-2 interactions but that addition of recombinant DMP1 had no such effect. Therefore, the invasiveness of SW480 colon cancer cells, which are known to express the partner protease of DMP1, MMP-9, but not MMP-2 (29) was measured using a modified Boyden chamber invasion assay. SW480 cells showed a clear dose-response increase in their invasiveness through the Matrigel barrier upon the addition of recombinant DMP1, with a maximum 3.9-fold increase at 100 nmol/L DMP1 (86.1 ± 13.9 versus 22.3 ± 9.8 for untreated cells, $P < 0.001$; Fig. 1). The increase in the invasiveness of the SW480 cell line by DMP1 was not due to enhancement of integrin-mediated cell attachment/migration alone because the same amount of recombinant human BSP or osteopontin, two other members of the SIBLING family that can support cell attachment/migration but cannot bind and activate MMP-9, did not increase the invasiveness of the SW480 cells (data not shown).

Dentin matrix protein 1–enhanced invasion requires matrix metalloproteinase-9 and cell surface receptors $\alpha_v\beta_3$ integrin, $\alpha_v\beta_5$ integrin, or CD44. Invasion enhancement studies were also done with DMP1-KAE, a recombinant DMP1 protein whose integrin-binding RGD sequence was replaced with the chemically
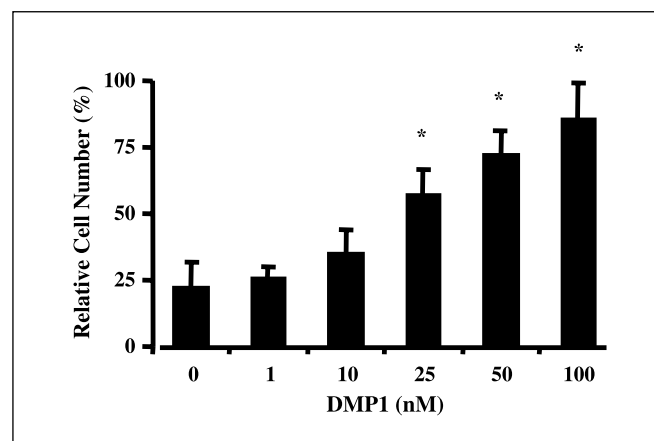

Figure 1. DMP1 and the invasion of the colon cancer cell line SW480 *in vitro*. Untreated or DMP1-treated SW480 colon cancer cells were placed in the top wells of separate Boyden chambers that each had Matrigel-coated UV-opaque transwell inserts. The lower chambers contained serum-free conditioned medium as a chemoattractant. The cells were incubated at 37°C for 24 hours. Invasive cells that penetrated the Matrigel artificial basement membrane barrier and moved into the lower chamber were then detected by calcein acetoxymethyl ester fluorescent dye activated by the live cells. The relative fluorescence in the lower chamber corresponded directly to the number of cells that digested their way through the barrier and migrated into to the lower chamber. *Columns,* means of triplicate samples; *bars,* 95% confidence intervals. $P < 0.001$ was obtained for multiple comparisons within the panel by use of one-way ANOVA. Each treatment group was also individually compared with the control (0 nmol/L DMP1) group by use of the Dunnett's test. $*P < 0.001$, compared with untreated cells by the Dunnett's test. All statistical tests were two-sided.

similar but inactive tripeptide, KAE. SW480 cells treated with DMP1-KAE showed a 2.4-fold increase at 100 nmol/L DMP1-KAE (83.6 ± 16.3 versus 34.4 ± 20.2 for untreated cells, $P < 0.01$; Fig. 2*A*). Although significantly less robust than the enhancement from normal DMP1, the mutant DMP1-KAE clearly aided the invasion by these cells without the ability to bind to RGD-dependent integrins. Addition of a blocking CD44 antibody completely inhibited the DMP1-KAE–enhanced invasion (12.7 ± 4.7 versus 11.1 ± 2.9; Fig. 2*B*), showing that DMP1 can act through both RGD-dependent integrins and CD44. To complete the study of cell surface proteins, blocking antibodies to the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (both RGD-dependent) as well as to CD44 were separately added to SW480 cells before the addition of normal DMP1 in the invasion assay. Each lowered the efficiency of the DMP1-enhanced invasion but not to DMP1-free control levels, suggesting that each was contributing to a portion of the DMP1 enhancement (Fig. 2*C*). Simultaneous addition of all three antisera reduced the DMP1-

enhanced invasion to control levels (Fig. 2*C*), suggesting that both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins contributed to the integrin-related DMP1 enhancement of invasion by SW480 cells.

Because integrins, CD44, and MMP-9 have previously been shown to be associated with cancer cell invasiveness (9, 16), and because DMP1 specifically binds to MMP-9 with nanomolar affinity (10), we hypothesized that DMP1 may bridge MMP-9 to the cell surface via CD44, $\alpha_v\beta_3$, and/or $\alpha_v\beta_5$ integrins where it could become more effective in digesting the Matrigel during the invasion process. Both DMP1- and DMP1-KAE–enhanced SW480 cell invasions were almost completely inhibited by the addition of a blocking antibody to MMP-9 (Fig. 2*D*).

Dentin matrix protein 1 bridges matrix metalloproteinase-9 to CD44, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins. Because DMP1 can separately form complexes with specific cell surface proteins and with MMP-9, DMP1 may be able to bridge MMP-9 to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins *in vitro*. The hypothesis was tested with pull-down experiments
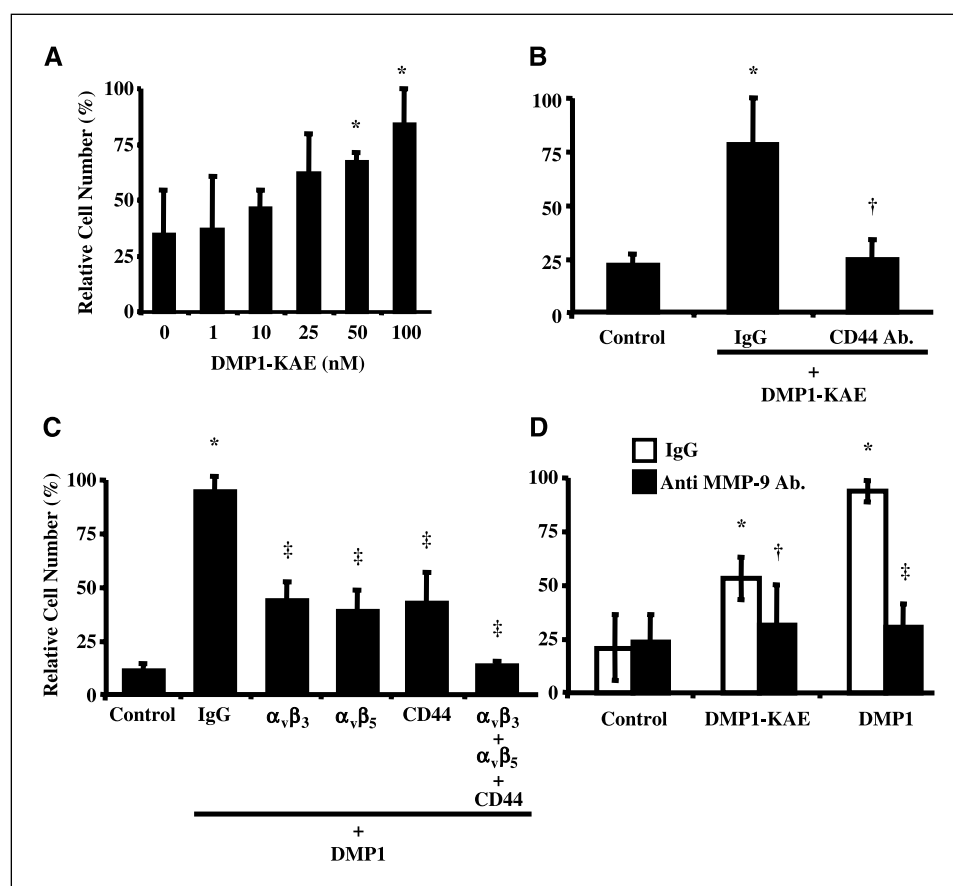

Figure 2. RGD-dependent integrins and CD44 are involved in the DMP1-enhanced invasion of colon cancer cells *in vitro*. *A,* SW480 cells treated with 0 to 100 nmol/L DMP1-KAE (in which the RGD domain of DMP1 was replaced with the integrin-inactive tripeptide, KAE) were placed in the upper portion of a modified Boyden chamber coated with Matrigel. The addition of DMP1-KAE resulted in a positive but less robust dose-response pattern compared with the integrin-binding native DMP1 (Fig. 1), showing that DMP1 does not have an absolute requirement to bind to RGD-dependent integrins to enhance invasion. *B,* addition of CD44 antibodies (*Ab.,* 20 μg/mL) to the DMP1-KAE treatment completely negates the enhanced invasion, showing that CD44 is the alternate cell surface–binding partner for DMP1. *C,* blocking mAbs to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, or CD44 (20 μg/mL of each antibody), were each able to partially block DMP1-enhanced invasion but not to control levels, suggesting that DMP1 can act through each of these cell surface receptors. Combined, the antibodies completely block the DMP1-enhanced invasion. Nonspecific IgGs had no affect on the invasion enhancement. *D,* inhibition of MMP-9 activity by addition of blocking antibodies (20 μg/mL) returned both the DMP1- and DMP1-KAE–enhanced invasion to control levels. Relative fluorescence, which corresponds to the number of cells that migrated through the Matrigel, is as described in Fig. 1. *A, columns,* mean of quadruplicate samples; *bars,* 95% confidence intervals. A *P* value of <0.01 was obtained for multiple comparisons within each panel by use of one-way ANOVA. Each treatment group was also individually compared with the control, untreated group by use of the Dunnett's test. *, $P < 0.01$, compared with untreated cells by the Dunnett's test. All statistical tests were two-sided. *B* to *D, columns,* mean of quadruplicate samples from a representative experiment; *bars,* 95% confidence intervals. A Mann-Whitney *U* test was used for the pairwise comparisons. *, $P < 0.01$, obtained for SW480 cells treated DMP1-KAE or DMP1 versus untreated respective control cells. $P < 0.01$, obtained for SW480 cells treated with antibody + DMP-KAE (†) or DMP1 (‡) versus isotype control IgG + DMP-KAE or DMP1, respectively. All statistical tests were two sided.
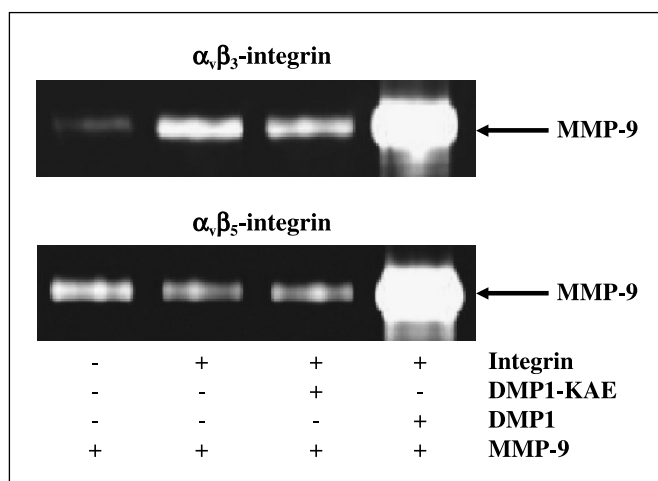
Figure 3. Pull-down experiments showing bridging of MMP-9 to $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin by DMP1. The $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins were first bound to their respective mAbs previously attached to beads by the manufacturer. After washing, the beads were incubated with buffer alone or buffer containing 500 nmol/L DMP1-KAE or 500 nmol/L DMP1, washed, and subsequently treated with recombinant proMMP-9. The washed samples were then electrophoresed on 10% zymogram gelatin gels and examined by Coomassie blue staining after digestion conditions were done. Beads alone have low background level of MMP-9 binding (*lane 1*). Note that the addition of DMP1 (*lane 4*) but not DMP1-KAE (*lane 3*) enabled proMMP-9 to be pulled down with both sets of integrin-bound beads. Control levels of proMMP-9 were observed without addition of DMP1 (*lane 2*).

using purified components. Only a small amount of proMMP-9 was detected using just the beads, showing that the pull-down assay had a low background (Fig. 3, *lane 1*). Beads with bound $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin but no DMP1 resulted in a similar, small amount of proMMP-9 pulled down with the beads (Fig. 3, *lane 2*). Addition of DMP1-KAE also gave background levels of bound proMMP-9 (Fig. 3, *lane 3*). However, addition of DMP1 to either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin–coated beads greatly increased the amount of bead-associated proMMP-9, indicating the bridging of proMMP-9 to each integrin (Fig. 3, *lane 4*). BSP and osteopontin are also known to be able to bind to $\alpha_v\beta_3$ and possibly $\alpha_v\beta_5$ integrins but substitution of recombinant BSP or osteopontin for DMP1 in the pull-down experiments resulted in only background levels of MMP-9, again illustrating the specificity of the DMP1 for proMMP-9 (data not shown). Because we were unable to obtain recombinant CD44, we could not test the formation of a proMMP-9/DMP1/CD44 complex *in vitro* using highly purified proteins in the pull-down experiments.

We were, however, able to detect proMMP-9/DMP1/CD44 complexes as well as proMMP-9/DMP1/integrin complexes on living cells through FACS analysis. Purified proMMP-9 was covalently labeled with the fluorescent dye, Alexa Fluor 488. Flow cytometry was used to determine the amount of labeled proMMP-9 bound to SW480 cells with and without DMP1 (or DMP1-KAE) treatment. Addition of DMP1 produced an 81% increase in the mean value of the signal of labeled proMMP-9 bound compared with that of cells not pretreated with DMP1 (Fig. 4A). The addition of DMP1-KAE produced a lower but significant increase of proMMP-9 binding (57%; Fig. 4B), verifying the earlier results showing that RGD-binding integrins were not the only cell surface protein that could be used by DMP1. Separate addition of blocking antibodies to $\alpha_v\beta_3$ integrin (Fig. 4D), $\alpha_v\beta_5$ integrin (Fig. 4E), or CD44 (Fig. 4F) before the treatment with DMP1 each partially blocked the DMP1-enhanced binding of the labeled proMMP-9 to SW480 cells. However, when cells were treated with the blocking

antibodies against both integrins and CD44 together, labeled proMMP-9 binding decreased to the DMP1-free control levels (Fig. 4G). Furthermore, when cells were first treated with anti-CD44 mAb and then with DMP1-KAE, MMP-9 binding was reduced to the untreated control levels (Fig. 4H), supporting the hypothesis of the formation of a MMP-9/DMP1/CD44 complex on cell surfaces.

Indirect fluorescent immunocytochemistry experiments were done to show that added DMP1 and MMP-9 both localize to the
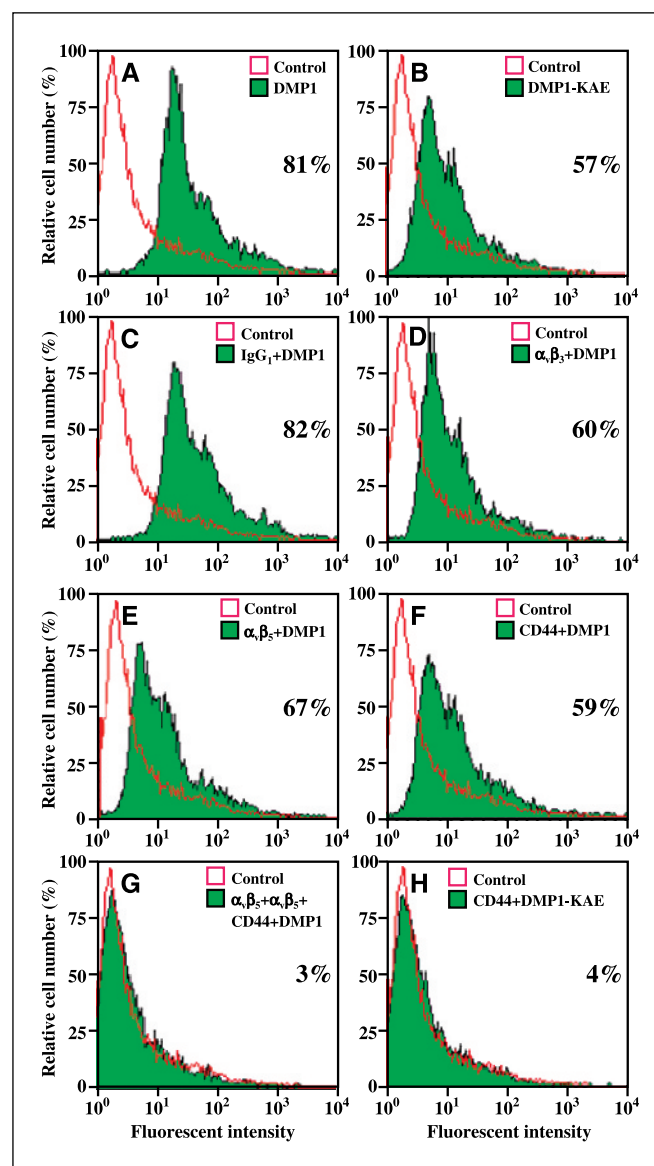

Figure 4. DMP1-enhanced binding of fluorescently labeled latent MMP-9 (proMMP-9) to SW480 cells. ProMMP-9 was labeled with Alexa Fluor 488 and incubated with cells treated as indicated or untreated cells (control). Pretreating the cells with DMP1 (*shaded area*) increased the amount of labeled proMMP-9 bound to the living cells, compared with untreated cells as analyzed by FACS (*A*). Pretreating cells with DMP1-KAE (*shaded area*) showed increased binding of labeled proMMP-9 compared with that of untreated cells (*open area*; *B*). Blocking cell surface molecules $\alpha_v\beta_3$ integrin (*D*), $\alpha_v\beta_5$ integrin (*E*), or CD44 (*F*) with their respective mAbs decreased the DMP1-enhanced binding of the labeled proMMP-9 but not to control levels. However, when all three antisera were added together ($\alpha_v\beta_3 + \alpha_v\beta_5 + $ CD44), the DMP1-enhanced binding of labeled proMMP-9 was completely blocked (*G*). Treating cells with a nonimmune IgG had no affect on the ability of DMP1 to enhance the binding of proMMP-9 (*C*). DMP1-KAE–enhanced binding of labeled proMMP-9 was essentially blocked by CD44 mAb alone (*H*). *Numbers,* percentage of cells bound to labeled proMMP-9.
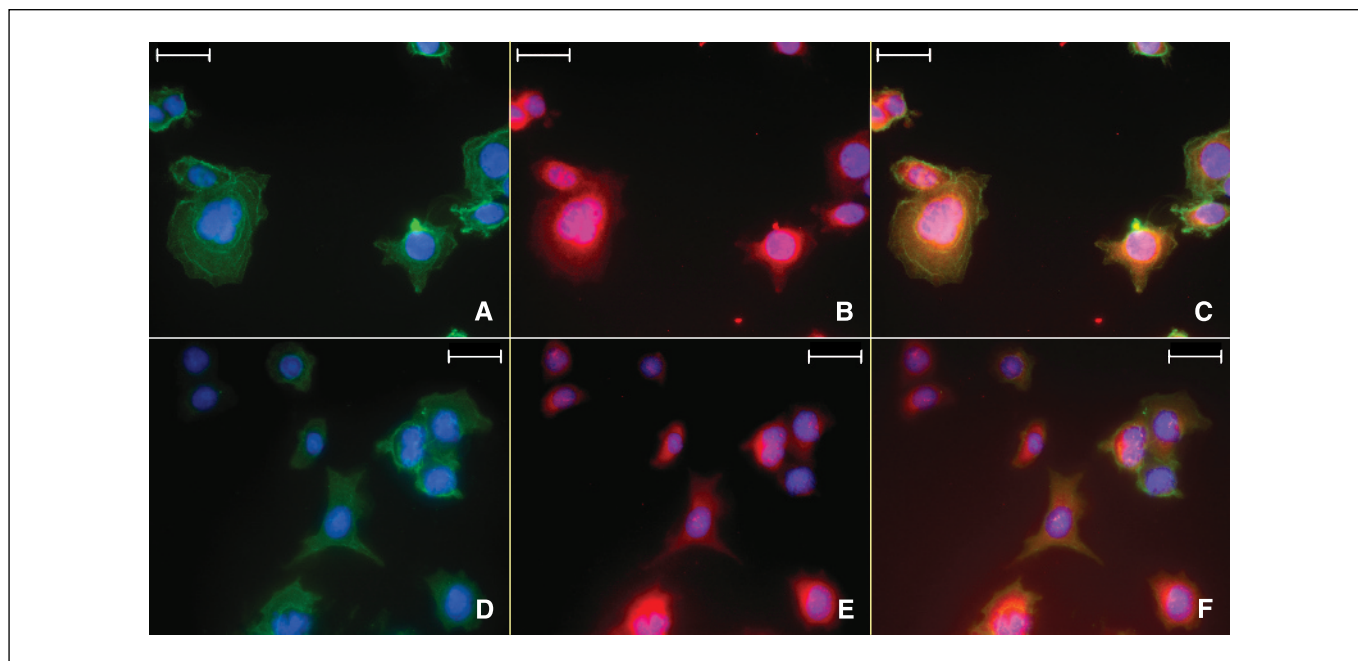
Figure 5. Colocalization of DMP1 and RGD-mutant DMP1 (DMP1-KAE) with MMP-9 on SW480 colon cancer cells. Cells were treated first with DMP1 and then with proMMP-9. The cells were incubated with a mouse mAb that can detect DMP1 as well as DMP1-KAE and an affinity purified rabbit polyclonal antibody against MMP-9. Bound antibodies were then detected by indirect immunofluorescence with Cy2-conjugated AffiniPure goat anti-mouse IgG for DMP1 or DMP1-KAE and Cy5-conjugated AffiniPure goat anti-rabbit IgG for MMP-9. *A, green,* location of DMP1. *B, red,* location of MMP-9. *C, yellow,* colocalization of DMP1 and MMP-9. *D, green,* location of DMP1-KAE. *E, red,* location of MMP-9. *F, yellow,* colocalization of DMP1-KAE and MMP-9. 4,6-Diamidino-2-phenylindole was used as a nuclear stain (*blue*). Bar, 20 μm.

same cells. DMP1 and MMP-9 were colocalized on the SW480 cells (Fig. 5*A-C*), suggesting that DMP1 rapidly localizes MMP-9 to the cell surface *in vitro* by binding to possibly both integrins and CD44 cell surface molecules. Added recombinant DMP1-KAE and MMP-9 were also detected at the same locations in these cells (Fig. 5*D-F*), suggesting that DMP1 also localizes MMP-9 to the cell surface *in vitro* through CD44. Like the SIBLINGs in most other established cancer cell lines, DMP1 was not detected in untreated SW480 cells but endogenous MMP-9 was weakly positive (data not shown).

Expression of dentin matrix protein 1, matrix metalloproteinase-9, CD44, and integrin subunits α_v, β_3, and β_5 in colon adenocarcinoma *in vivo*. The expression of MMP-9, CD44, as well as the α_v, β_3, and β_5 integrin subunits, in tumors have been shown separately and in some combinations many times previously. However, to illustrate that the MMP-9/DMP1/cell surface receptors are made by the same cells and, therefore, logically may form the complexes describe above *in vivo*, immunohistochemistry reactions were done on serial paraffin sections of a human colon adenocarcinoma. The results show that all six proteins are coexpressed in the same cells/areas, suggesting that the complexes could easily be locally formed by simple interactions (Fig. 6). The red/brown staining indicates the expression of DMP1 (Fig. 6*A*), CD44 (Fig. 6*B*), α_v integrin subunit (Fig. 6*C*), β_3 integrin subunit (Fig. 6*D*), β_5 integrin subunit (Fig. 6*E*), and MMP-9 (Fig. 6*F*). The negative controls, mouse IgG (Fig. 6*G*), and rabbit nonimmune serum (Fig. 6*H*) illustrate the low background for these assays.

Discussion

Cancer progression reaches a critical period when a tumor starts to invade and metastasize. One of the initial steps in metastasis requires invasion of tumor cells through basement membrane and nearby stroma. The presence of MMPs, various cell adhesion molecules, and SIBLING proteins during tumor development, growth, invasion, metastasis, and angiogenesis have been well documented. However, the mechanisms by which these molecules work in the coordination of tumor progression are less well elucidated. Several colocalization studies have reported that $\alpha_v\beta_3$ integrin may function not only as an adhesion and/or migration receptor but also to activate and properly distribute proteases that degrade the extracellular matrix during invasion (23, 24, 30). Three members of the SIBLING family (BSP, osteopontin, and DMP1) bind and activate proMMP-2, proMMP-3, and proMMP-9, respectively (10). Furthermore, the same pairings of SIBLINGs and active MMPs cause their respective TIMPs to be much less effective at inhibiting the proteases. Recombinant BSP has been shown to promote invasion of many MMP-2-expressing osteotropic cancer cell lines through formation of a MMP-2/BSP/$\alpha_v\beta_3$ integrin complex (18). DMP1, which has recently been shown to be expressed by various tumors (8, 9), had no effect on these same MMP-2-producing cell lines.

In this article, the role of DMP1 in an invasion assay was studied using a colon cancer cell line, SW480, that has been reported to express significant levels of MMP-9 but no MMP-2 (29). Recombinant DMP1 added to the Boyden chamber wells caused a dose-response increase in the invasiveness of the cells through a Matrigel barrier. Addition of two other SIBLING family members with similar cell attachment properties, BSP or osteopontin, did not increase the invasiveness of the SW480 cells suggesting that the action of DMP1 was not due to simple attachment and/or migration effects. This result is similar to the specific enhancement of invasion of several MMP-2-expressing cancer cell lines in

response to the addition of the SIBLING partner of MMP-2, BSP, but not to either osteopontin (partner of MMP-3) or DMP1. Negating the ability of DMP1 to bind to RGD-dependent integrins by mutation of the RGD to the inactive KAE or by the addition of antibodies that block $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin only partially affected the ability of DMP1 to enhance invasion. This suggests that DMP1 may also be using another of its known cell surface receptors, CD44. Antibodies to CD44 resulted in a partial block in the DMP1-enhanced invasion and a complete return to control levels when used with the DMP1-KAE protein. Used together, antibodies against $\alpha_v\beta_3$ integrin, $\alpha_v\beta_5$ integrin, and CD44 reduced the DMP1-enhanced invasion to nearly control levels.

The DMP1-enhanced invasion by the SW480 cells was also inhibited by a specific antibody for it protease partner, MMP-9. Pull-down studies using purified proteins showed the formation of MMP-9/DMP1/$\alpha_v\beta_3$ integrin and MMP-9/DMP1/$\alpha_v\beta_5$ integrin complexes *in vitro*. Because purified recombinant CD44 was not available, we did not look at the formation of the hypothesized MMP-9/DMP1/CD44 complex by this approach. However, the formation of the DMP1/MMP-9/CD44 as well as the other two integrin-related complexes at the cellular level by FACS analysis was shown. Together, these results suggest that cells use DMP1 as a bridge to link MMP-9 to its cell surface receptors, $\alpha_v\beta_3$ integrin, $\alpha_v\beta_5$ integrin, and/or CD44, thereby increasing their ability to invade through basement membranes and other connective tissues particularly in the absence of MMP-2. The modified Boyden chamber assay can measure only certain rate-limiting steps in this model system but one can hypothesize that *in vivo*, a cell may use a BSP/MMP-2/integrin complex to digest some matrix components and a DMP1/MMP-9/integrin or DMP1/MMP-9/CD44 complex to digest other proteins during the metastasis process. This may help to explain why some invasive tumors are found to express a combination of SIBLINGs and their MMP partners.

Figure 6. The expression of DMP1, MMP-9, CD44, and all three subunits of integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in near-serial sections of moderately differentiated colon adenocarcinoma. Deparaffinized sections of a colon adenocarcinoma were incubated separately with antibodies specific for DMP1, MMP-9, CD44, and the α_v, β_3, and β_5 integrin chains. Localization of the antibodies was determined with SuperPicture Polymer HRP-conjugated broad-spectrum secondary antibody and AEC Single Solution chromogen. *Red/brown color,* DMP1 (*A*), CD44 (*B*), α_v integrin (*C*), β_3 integrin (*D*), β_5 integrin (*E*), and MMP-9 (*F*) are all associated with the same cells, suggesting that all proteins required for formation of complexes of CD44, or integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$, DMP1, and MMP-9, are expressed in the same cells. The negative controls, mouse IgG (*G*), and rabbit immune serum (*H*), revealed no signal. Mayer's hematoxylin was used as a nuclear counterstain (*purple blue*). Bar, 100 μm.
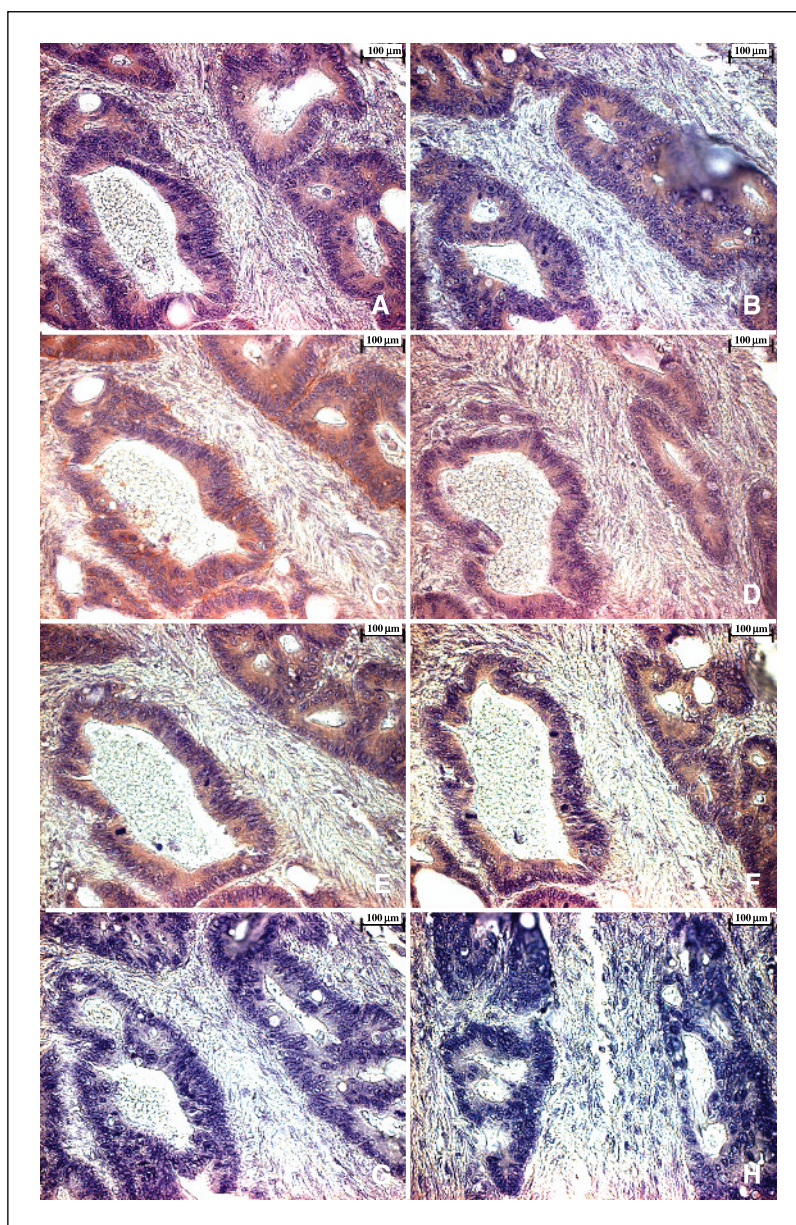
In conclusion, it was shown that DMP1 can enhance the invasiveness of a model colon cancer cell *in vitro* by bridging MMP-9 to the cell surface in an RGD-dependent (with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin) and RGD-independent (with CD44) manner. Furthermore, it was shown that intact tumors can express all of these proteins within the same cellular structures, suggesting that these complexes studied *in vitro* will likely be formed *in vivo*.

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